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Engineering and characterization of a humanized antibody targeting TNF-α and RANKL

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ABSTRACT

To neutralize the pathological activities of tumor necrosis factor-α (TNF-α) and receptor activator of NF-κB ligand (RANKL), we engineered and characterized a humanized 8G12 (h8G12) antibody that targeted TNF-α and RANKL. Standard molecular biological and complementarity determining region (CDR)-grafting techniques were used to engineer the h8G12 antibody, and enzyme-linked immunosorbent assays (ELISAs) and Western blotting were employed to determine its binding activation and specificity. TNF-α-mediated cytotoxicity and RANKL-induced osteoclastogenesis assays were used to evaluate the neutralizing effects of the antibody. The cDNA sequences were established by grafting the murine monoclonal antibody (mAb) 8G12 CDRs into the heavy and light chain (HC and LC) variable regions (VH and VL) of the human mAbs 3DGG_B and 1I9R_L, respectively. The recombinant plasmids were transfected into Chinese hamster ovary (CHO) cells to produce the h8G12 antibody, which could simultaneously recognize TNF-α and RANKL. In addition, the h8G12 antibody reduced the TNF-α-mediated apoptosis of L929 cells by 25.84%. Furthermore, the h8G12 antibody significantly inhibited leukocyte infiltration in a murine allergic contact inflammation model. Concurrent with the inhibition of apoptosis, the h8G12 antibody significantly reduced the number of osteoclast-like cells in a dose-dependent manner. These results demonstrated that the h8G12 antibody neutralized the activities of TNF-α and RANKL and that it might be a potential candidate for the treatment of inflammatory bone diseases, such as rheumatoid arthritis (RA).

1. Introduction

Rheumatoid arthritis (RA) is a chronic and symmetric polyarthritis that is characterized by protracted inflammatory infiltration of the synovial membrane associated with the destruction of cartilage and underlying bone [1]. The accumulation of inflammatory cells and their secretions in joints attracts osteoclasts and their precursor cells, leading to further cartilage erosion and bone loss [2]. Tumor necrosis factor-α (TNF-α) and receptor activator of NF-κB ligand (RANKL) are abundant at sites of synovitis, which are known to promote osteoclast recruitment, differentiation, and activation [3,4]. The role of TNF-α in inflammatory diseases demonstrates that it is an early and crucial cytokine that triggers downstream mediators and forms a variety of positive and negative feedback loops to exacerbate inflammatory disorders [5]. TNF-α also enhances RANKL secretion, and high levels of RANKL induce osteoclast formation, bone erosion and breakage in arthritis [6]. Thus, TNF-α and RANKL may orchestrate bone destruction and joint inflammation in RA. Moreover, intervention studies have shown that the combined treatment of blocking TNF-α-mediated inflammation and RANKL-induced osteoclast activation had more prominent salutary effects that fully suppressed inflammation and prevented bone destruction in arthritic models and in the clinic [7].

Over the last decade, using antibodies as a treatment option for RA has been widely accepted as a viable therapeutic approach by patients, clinicians, regulatory agencies and the pharmaceutical industry [8]. As members of the TNF superfamily, TNF-α and

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RANKL share some similarities in structure and play a central role in aggravating synovitis and bone destruction. In our previous studies, we engineered a dual-function vaccine, RTFP-2, using two segments of the TNF-like domain of RANKL. The RTFP-2 vaccine, which simultaneously targets TNF-α and RANKL *in vitro* and *in vivo*, ameliorated the symptoms of collagen-induced arthritis (CIA) in mice [9]. Subsequently, we screened for a positive anti-RANKL/TNF-α hybridoma cell (ARTHC) line that secreted the monoclonal antibody (mAb) 8G12, which could neutralize TNF-α- and RANKL-mediated bioactivities, thus mitigating acute inflammation and osteoporosis [10]. However, the utilization of mouse-derived antibodies in patients often caused the human anti-mouse antibody (HAMA) response, resulting in rapid antibody clearance, loss of efficacy and hypersensitivity reactions [11].

To reduce immunogenicity, chimeric, humanized, and fully human antibodies were designed using increasing amounts of human sequence. However, the chimeric antibodies still elicited an undesirable antivariable region response [12]. As molecular biology technology developed, it became possible to further reduce the immunogenicity of the chimeric antibodies by replacing murine variable region frameworks (FRs) with those of the selected human antibodies using a "complementarity-determined region (CDR)-grafting" approach [13]. This method produced humanized antibodies containing 85–95% human sequences. Numerous clinical studies have confirmed that humanized antibodies are less immunogenic and more therapeutic in humans than murine or chimeric antibodies [14]. Therefore, in the present study, the CDRs from the heavy and light chain (HC and LC) variable regions (VH and VL), which form antigen-binding sites, of the murine mAb 8G12 were grafted into antibody FRs of selected, homologous human antibody variable regions, and some mouse residues among the FRs were retained to conserve the conformation of the CDRs. The humanized 8G12 (h8G12) antibody VH and VL genes were then inserted into commercial plasmids containing human HC and LC constant regions (CH and CL) to construct the humanized antibody gene. After expression and purification, the recombinant h8G12 antibody (~90% human sequences) retained its conserved antigen-binding specificity and therapeutic efficacy. The h8G12 antibody reduced the TNF-α-mediated apoptosis of L929 cells by 25.84%. In addition, the h8G12 antibody significantly inhibited leukocyte infiltration in a murine allergic contact inflammation model. Concurrent with the inhibition of TNF-α bioactivities, the h8G12 antibody completely abolished RANKL-induced osteoclast differentiation. These results demonstrated that the h8G12 antibody might be a potential candidate for the treatment of inflammatory bone diseases, such as RA.

2. Materials and methods

2.1. Mice

Female BALB/c mice (6–8 weeks old) were purchased from Beijing HFK Bio-technology Co., Ltd. All mice were housed in a controlled environment and allowed food and water *ad libitum*. All experiments were conducted in accordance with the requirements of the Animal Care and Use Committee of Capital Medical University (Permit ID: 2009-X-870).

2.2. Cloning the mouse variable region gene

The experiments were performed according to the manufacturer's instructions. Total RNA was isolated from a mouse hybridoma cell line (ARTHC) [10] using a SV Total RNA Isolation System (Promega BioSciences, CA, USA) and the cDNA was amplified with a First Strand cDNA synthesis kit (Merck KGaA, Darmstadt, Germany). The Go Taq® Colorless Master Mix (Promega) was used to amplify the VL and VH cDNAs of this hybridoma cell line using primers specific for mouse antibodies, as previously described [12]. Then, the PCR products for the variable region cDNA were amplified using the following conditions: 35 cycles at 94 °C for 30 s (denaturation), 55 °C (VL) or 58 °C (VH) for 30 s (annealing), 72 °C for 1 min (extension), and a final 10-min extension at 72 °C.

2.3. Designing the humanized antibody Fv genes

The humanization of ARTHC Fv genes was performed by CDR-grafting as described previously with minor modification [15]. Briefly, to select human germline V genes as FR donors (FRs 1–4), mouse VH and VL DNA sequences were separately subjected to a VBASE2 database search to locate the CDR and FR loci [16]. Then, genes similar to the human germline variable regions (CDRs 1–3 for HC and the LC variable regions) and ARTHC were considered FR donors. Finally, CDR genes of the ARTHC variable regions were grafted into the FR genes of the V regions of the selected human germline donor, resulting in humanized Fv genes [17].

2.4. Construction, expression and purification of the humanized antibody

The humanized VH and VL DNA sequences were separately cloned into the pFUSE2ss-CHIg-hG1 and pFUSE2ss-CLIg-hκ plasmids, resulting in the recombinant plasmids pFUSE2ss-CHIg-hG1-hHV and pFUSE2ss-CLIg-hκ-hLV, respectively (Fig. 1S) [18]. The recombinant plasmids were co-transfected into Chinese hamster ovary (CHO) cells using the TurboFect Transfection Reagent (ThermoScientific, Pittsburgh, PA, USA), and the h8G12 antibody was expressed. The expression of the h8G12 antibody by all clones was assayed by sandwich enzyme-linked immunosorbent assays (ELISAs). The best-expressing clones were then amplified to produce the h8G12 antibody, and the h8G12 antibody was purified from the culture media using a HiTrap Ig select protein G chromatography column (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

2.5. Identification of the humanized antibody

First, the expression of the h8G12 antibody was detected using Western blotting. Briefly, the h8G12 antibody was separated in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, USA). The membrane was incubated with affinity purified HRP-conjugated goat anti-human IgG (H + L) antibody (KPL) for 1 h at room temperature. The target protein was examined using the SuperSignal® West Pico ECL system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and visualized with autoradiography film. Then, the humanized antibody was classified using a sandwich ELISA. Briefly, 96-well plates were coated with goat anti-human κ chain-specific capture antibody (200 ng/well, Southern Biotech) overnight at 4 °C. Supernatant (200 μl) from transfected cells expressing humanized antibody was added to the wells and incubated overnight at 4 °C. After washing, the HRP-conjugated goat anti-human IgG_1 Fc-specific detection antibody (100 μl, diluted 1:2000, Southern Biotech, Birmingham, AL, USA) was added, and the plates were incubated for 1 h at 37 °C. OD_{450} readings were measured using an ELISA plate reader.

2.6. Binding determination of the humanized antibody

Recombinant human RANKL or TNF-α (200 ng, R&D Systems) were added to wells of a 96-well plate and incubated at 4 °C

overnight. Humanized 8G12 or human RANKL or TNF-α antibodies (1:2000 dilution, R&D Systems) were added to each well and incubated overnight at 4 °C. After washing, 100 μl of affinity purified, HRP-conjugated goat anti-human IgG (H + L) antibody (1:1000; KPL, Gaithersburg, MD, USA) or rabbit anti-goat polyclonal secondary antibody IgG-H&L (HRP) (1:100,000; Abcam, Cambridge, MA, USA) was added to each well, and the plate was incubated at 37 °C for 1 h. OD_{450} readings were measured using an ELISA plate reader.

Western blotting was utilized to detect the capability of the recombinant antibody to bind to RANKL and TNF-α. Recombinant human RANKL and TNF-α were separated by 10% SDS–PAGE and transferred to PVDF membranes. Then the membrane was incubated with recombinant antibody for 2 h and HRP-conjugated goat anti-human IgG (H + L) antibody (KPL) for 1 h at room temperature. The target protein was examined as described above.

2.7. Cytotoxicity assay

The cytotoxicity assay was assessed as previously described [19]. Briefly, murine fibroblast L929 cells (10^5 cells/well) were seeded in 96-well plates and incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) for 16 h. Subsequently, the cells were treated with 0.25 ng/ml TNF-α (R&D Systems) and 1 μg/ml actinomycin D (Sigma–Aldrich, Saint Louis, MO, USA) with or without infliximab (Cilag AG, Schaffhausen, Switzerland) or diluted recombinant h8G12 antibody for 12 h. After removing the supernatant, the cells were stained with crystal violet for 20 min, washed with ddH_2O and dissolved with acetic acid. The resulting optical density was measured at 570 nm using a complete GloMax-Multi detection system (Promega).

2.8. Osteoclastogenesis assay

An *in vitro* osteoclastogenesis assay was performed as described previously [20]. Bone marrow cells were obtained by flushing the marrow spaces of tibias and femurs from 6-week-old BALB/c mice with α-minimal essential medium (α-MEM) containing 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma–Aldrich). The cells were cultured for 16–24 h with M-CSF (5 ng/ml, R&D Systems) and nonadherent cells were completely removed by aspiration. The adherent cells were utilized as osteoclast precursor cells. To achieve osteoclast differentiation, the cells were seeded in 24-well glass insert plates at 5×10^5 cells/well. The cells were cultured for 6 days with M-CSF (30 ng/ml) and RANKL (40 ng/ml) in the presence or absence of goat anti-human RANKL (0.1 μg/ml) or h8G12 antibody (0.1 μg/ml, 1 μg/ml or 10 μg/ml). An acid phosphatase kit (Sigma Aldrich) was utilized to demonstrate tartrate-resistant acid phosphatase (TRAP) in the cultured cells.

2.9. Contact hypersensitivity model

DNFB (1-Fluoro-2,4-dinitrobenzene, Merck, Schwalbach, Germany) was diluted in ethanol (100%) immediately before use [21]. Briefly, 8-week-old BALB/c mice were sensitized by painting 40 μl of 0.2% DNFB on the shaved abdomen for two consecutive days. The mice were challenged by painting both sides of one ear with 10 μl of 0.2% DNFB on day 5. Twenty-four hours before painting, h8G12 (200 μg/injection, intraperitoneally) was applied 5 times daily. Paraffin-embedded ear tissue was stained with hematoxylin and eosin, and the number of inflammatory cells in high-power fields (HPF) per cm^2 was calculated.

2.10. Statistical analysis

The data were analyzed using a statistical package (SPSS13.0, Chicago, IL, USA). The Student's *t*-test or one-way analysis of variance for parametric analysis was utilized for between-group comparisons. *p* values less than 0.05 were considered statistically significant.

3. Results

3.1. CDR-grafts of mouse variants

The lengths of the amplified mouse variable sequences for the LC and HC regions were ~400 base pairs (bp). The identified Ig CDRs comprised residues 26–33, 51–58, and 97–108 in the VH and residues 27–36, 54–56, and 93–104 in the VL. To select human germline antibody V genes as FR donors for the humanization of mouse variants, those with the most similar CDRs and key residues of the FRs were chosen as FR donors 1–3. In fact, in the HC, there were only four differences in ten residues of CDR1, two differences in five residues of CDR2, and seven differences among twelve residues of CDR3. For the LC, ten residues of the murine CDR were replaced with human residues. The sequences of the murine variable regions were analyzed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the VH (3DGG_B) and VL (1I9R_L) of the most homologous, humanized antibody, and the sequences shared 92.44% and 82.88% identity (Fig. 1).

3.2. Expression and identification of a humanized antibody

To obtain the secreted h8G12 antibody, the VH and VL sequences were synthesized and cloned into the pFUSE2ss-CHIg-hG1 and pFUSE2ss-CLIg-hκ vectors, respectively, with leader sequences for expression (Fig. 1S). High titers of humanized antibodies were found in the supernatants at 48 h by sandwich ELISA (Fig. 2A). Western blotting further showed that the secreted antibody was humanized. Two protein bands of approximately 23 kDa and 50 kDa were observed (Fig. 2B).

Subsequently, six cell clones were selected for stable production of the h8G12 antibody, and the highest-producing clone was cultured for antibody production. After purification and condensation, the final concentration of the purified recombinant protein was 2 mg/ml, and the purity was greater than 90% (Fig. 2C). Western blotting showed that the h8G12 antibody could simultaneously recognize RANKL and TNF-α (Fig. 2D). In addition, ELISA assays showed that the h8G12 antibody was dually reactive to RANKL and TNF-α, whereas the commercial RANKL and TNF-α antibodies only bound a specific protein (Fig. 2E). To verify the binding activity of the h8G12 variants, indirect ELISA showed that the affinity of h8G12 for its ligands was increased in a dose-dependent manner (Fig. 2F). Furthermore, the recognition of h8G12 was also specific (Fig. 2G).

3.3. The biological function of the humanized antibody

To evaluate the effects of the h8G12 antibody in suppressing inflammation, the TNF-α-mediated cytotoxic assay was utilized. The data showed that TNF-α induced more than 47.85% apoptosis in L929 cells, and treatment with the h8G12 antibody reduced the apoptosis level in a dose-dependent manner (Fig. 3A). In addition, the inhibitory effect was comparable to that of infliximab, a commercially available mAb to TNF-α (Fig. 3B). To further evaluate the anti-inflammatory effects of the h8G12 antibody, we used a murine allergic contact inflammation model. Compared with the

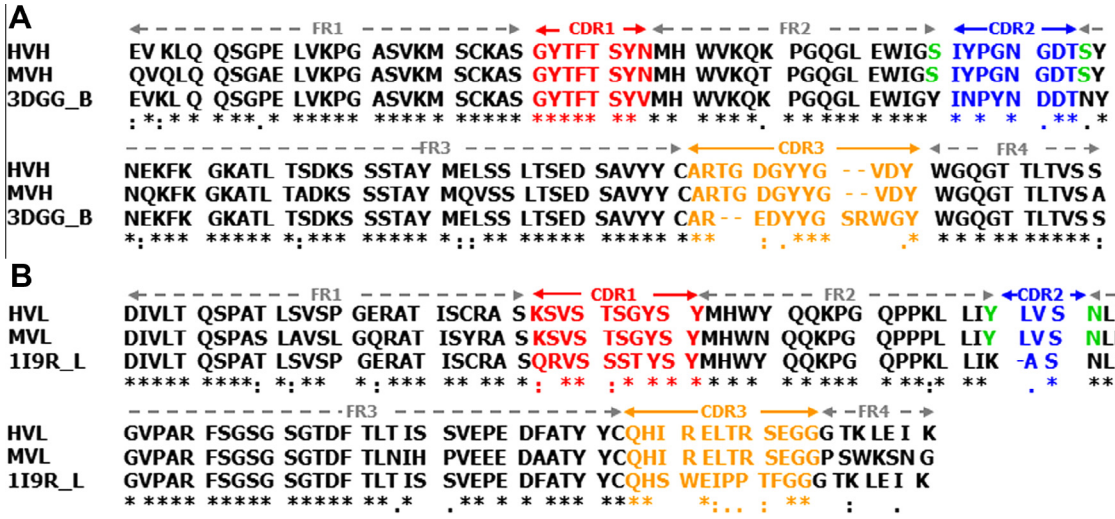

Fig. 1. Sequence alignment of the variable regions of mice and humans. (A) Sequence alignment of the heavy chain variable region. HVH, human heavy chain variable region; MVH, mouse heavy chain variable region. (B) Sequence alignment of the light chain variable region. HVL, human light chain variable region; MVL, mouse light chain variable region.

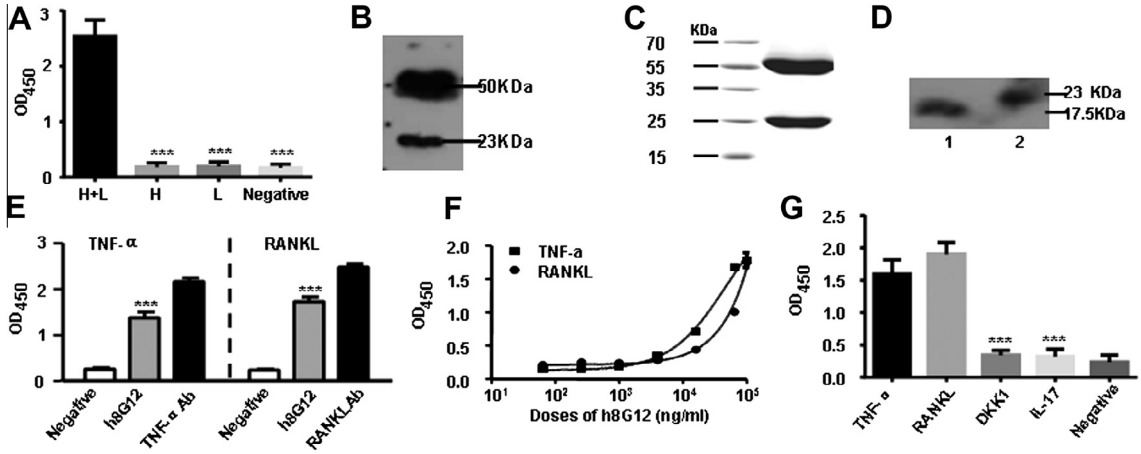

Fig. 2. Identification and binding affinity determination of the h8G12 antibody. (A) ELISA analysis of the h8G12 antibody. (B) Western blotting of the h8G12 antibody. (C) Purity analysis of the h8G12 antibody by SDS–PAGE. (D) Recognition analysis of the h8G12 antibody by Western blotting. (E) Binding analysis of the h8G12 antibody by ELISA. (F) Affinity analysis of the h8G12 antibody by ELISA. (G) Specificity analysis of the h8G12 antibody by ELISA. The data are expressed as the means ± SEM, $N = 10$/group. $^{***}p < 0.001$.

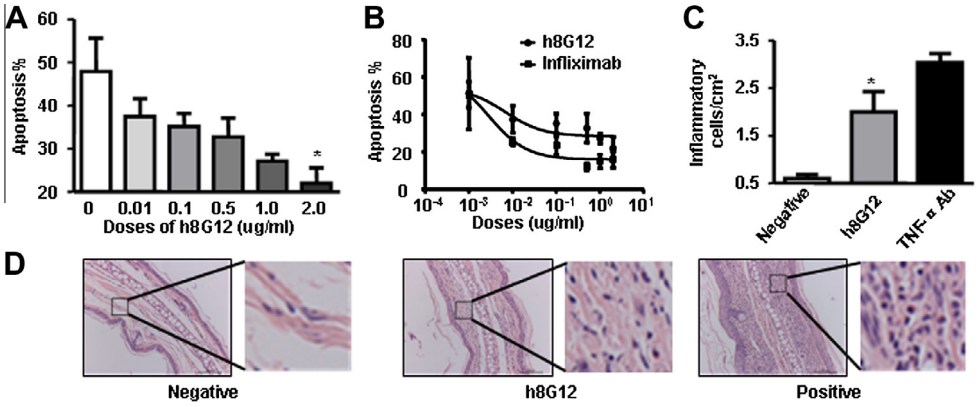

Fig. 3. Neutralization of TNF-α bioactivity by the h8G12 antibody. (A and B) The murine fibroblast cell line L929 ($N = 9$/group) was treated with TNF-α and various concentrations of h8G12 or infliximab for 12 h. Crystal violet staining was used to calculate the apoptosis of the L929 cells. The optical density was measured at 570 nm. (C and D) Female BALB/c mice ($N = 10$/group) were sensitized by painting DNFB on the shaved abdomen and then treated intraperitoneally with h8G12. The images were obtained, and the number of inflammatory cells was calculated in 1-cm^2 high-power fields (HPF). The bar indicates 100 μm. The data are expressed as the means ± SEM. $^{*}p < 0.05$.

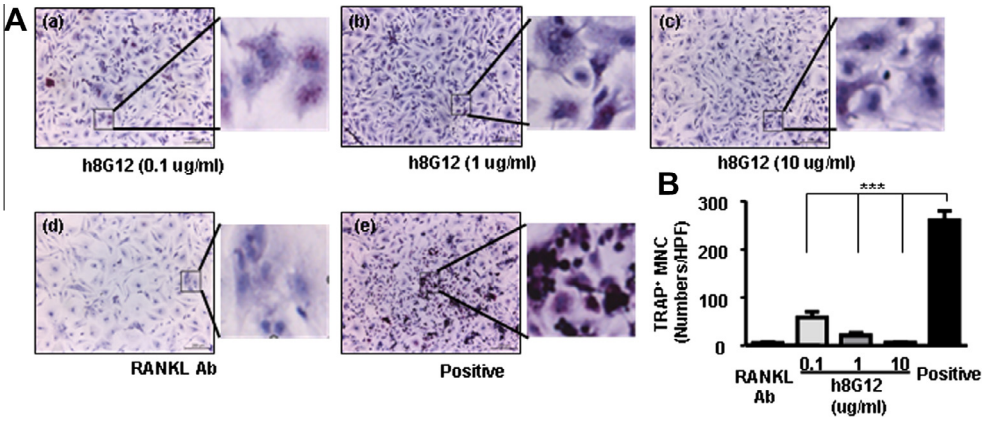

Fig. 4. Neutralization of RANKL bioactivity by the h8G12 antibody. Bone marrow cells were cultured with M-CSF (30 ng/ml) and RANKL (40 ng/ml) in the presence or absence of goat anti-human RANKL (0.1 μg/ml) or the h8G12 antibody (0.1 μg/ml, 1 μg/ml or 10 μg/ml). TRAP staining was performed to identify mature osteoclasts. The images (A) were obtained, and TRAP-positive cells with more than one nucleus were counted (B). The bar indicates 200 μm. The data are expressed as the means ± SEM, $N = 5$/group. $^{***}p < 0.001$.

positive controls, the numbers of inflammatory cells were significantly decreased in local areas (Fig. 3C and D).

To further assess the inhibition of RANKL-mediated osteoclast differentiation by the h8G12 antibody, variable concentrations of h8G12 were added to preosteoclasts. As expected, osteoclastogenesis assays showed that the h8G12 antibody significantly reduced the number of osteoclast-like cells in a dose-dependent manner (Fig. 4A and B). Taken together, these results suggest that the h8G12 antibody not only markedly suppresses TNF-α-mediated pathologies but also inhibits RANKL-induced osteoclast differentiation.

4. Discussion

In this study, we generated a humanized antibody, named h8G12, by a CDR-grafting approach to overcome the therapeutic deficiencies of mouse monoclonal antibodies. This methodology has been proven to be effective in retaining antigen recognition and reducing immunogenicity [13]. To obtain the complete sequence of the murine antibody variable regions, we analyzed the functional residues of the CDRs and removed the leader sequences [22]. Variable regions were constructed by grafting murine monoclonal antibody CDRs of the light or heavy chains to the CDRs of the corresponding human antibody. To select the most appropriate human frameworks for CDR grafting, murine mAb sequences that were highly homologous to the human antibody 1I9R_L and 3DGG_B regions were chosen. There were only 21 differences in 119 amino acid residues between the heavy chains of mAbs 8G12 and 3DGG_B. The CDR grafting approach, which consists of the transfer of murine CDRs to human frameworks, frequently results in a significant loss of antigen-binding affinity. It is known that certain framework residues are critical for preserving the conformation of the CDRs or are directly involved in antigen binding. In most cases, the successful design of humanized antibodies requires these key murine residues to be reintroduced into the human frameworks to restore affinity [23]. To avoid the replacement of such residues, we retained the amino acid residues near the CDR2 of the murine antibody (Fig. 1), which maintained the binding affinity of the humanized antibody Fab. In fact, our data showed that the affinity of h8G12 for its ligands was increased in a dose-dependent manner and that the recognition by h8G12 was specific.

TNF-α may stimulate differentiation of bone marrow macrophages into osteoclasts in a RANKL-RANK-dependent manner. Moreover, the secretion of RANKL by activated inflammatory cells further enhances the effects of cytokines such as TNF-α and IL-1, which promote inflammation and bone resorption [24]. The close association between TNF-α and RANKL may possibly explain why TNF inhibitors or anti-RANKL antibodies alone fail to completely inhibit inflammatory and bone damage. Consistent with other studies, our previous studies demonstrated that the dual-targeting RTFP-2 vaccine, which binds TNF-α and RANKL, exhibited a more obvious therapeutic effect than that of infliximab in CIA mice [9]. In this study, simultaneous binding of the h8G12 antibody to TNF-α and RANKL demonstrated that it might be a good candidate for treatment of RA.

At the equal curative dosage, it is difficult for the h8G12 antibody to exhibit the identical action as the commercial TNF-α or RANKL antibodies, but these differences are significantly curtailed with increasing dosage. In future studies, we will alter the sequences of the h8G12 antibody variable regions to increase its affinity for TNF-α and RANKL. These studies will help make the h8G12 antibody, which targets both TNF-α and RANKL, a potential therapeutic antibody for the treatment of RA.

In conclusion, we engineered a humanized antibody using CDR-grafting techniques to neutralize the bioactivities of TNF-α and RANKL. Our data demonstrated that the humanized antibody might be a potential candidate for inflammatory bone diseases, such as RA.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.046.

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